United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health and Science

SOP No: CLG-AVR1.00		Page 1 of 17
Title: Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometric (LC/APCI/MS) Confirmation of Ivermectin, Doramectin and Moxidectin.		
Revision: .00	Replaces: NA	Effective: 2-24-03

Contents

Approval Signatures on file

A. INTRODUCTION

1. Theory

   This method is used to confirm residues of ivermectin, doramectin and moxidectin. The acetonitrile extract obtained from the determinative method (CLG-AVR) prior to derivatization for LC analysis is further purified by using a C8 solid phase extraction (SPE) cartridge and an alumina-B SPE cartridge. The purified extract is analyzed by injecting into an LC/APCI mass spectrometer. The identity of the compound is confirmed by comparison of its retention time and relative intensity data with those of a standard or a recovery. Methanol extracts of liver and muscle containing ivermectin, doramectin, and moxidectin at 25 ppb levels were successfully confirmed by this procedure.

2. Applicability

   Tissues/species of interest are liver and muscle in bovine, ovine, porcine, caprine and equine species.

B. EQUIPMENT

Note: Equivalent apparatus or instrumentation may be substituted for any of the following.

1. Apparatus

   a. Amber ABC screw-top vials - 2 mL Cat. No. 27331, Supelco, Bellefonte, PA.

   b. EDP Plus Micropipets - 250 μL capacity, Rainin Instrument Inc. Emeryville, CA.

   c. Mechanical shaker - Eberbach model 610 equipped with shaker box model 6040. Thomas Scientific, Swedsboro, NJ 08065-0099.

   d. Vortex mixer - Fisher Scientific, Fisher Scientific, Norcross, GA 30091.

   e. 50-mL polypropylene centrifuge tubes - Cat. No. 2098, Becton Dickerson Labware, 2 Bridgewater Lane, Lincoln Park, NJ 07035.

   f. 16-port Vacuum manifold - Altech Associates, Inc., 2051 Waukegon Rd, Deerfield, IL 60015.

   g. 15-mL disposable glass conical centrifuge tubes with snap caps, VWR Cat No. 21020.

   h. N-Evap - Model 112, Organomation Assoc. Inc., Berlin, MA 01503

i. C8 Bond Elut cartridge - Part No. 1210-2002, Varian, 24201 Framton Ave, Harbor City, CA 90710.

j. Sep-Pak Plus Alumina B Cartridge - Part No. WAT 020505, Waters Corporation, 34 Maple St., Milford, MA 01757.

2. Instrumentation

a. LC/APCI MS - Waters 2690 LC with Waters/Micromass QA MS

i. LC column - Waters Nova-Pak 15 cm x 2 mm id. C-18, 4μm particles with an Optigard 1mm. C-18 guard column. LC column temperature at approximately 23ºC.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted for any of the following.

1. Reagents

a. Acetonitrile (ACN) - HPLC grade.

b. Methanol - HPLC grade.

c. Water - HPLC grade.

d. Hexane - HPLC grade.

e. Triethylamine - HPLC grade.

f. Methylene chloride - HPLC grade.

g. Ethyl acetate - HPLC Grade.

2. Solutions

a. SPE wash solution - ACN + water + triethylamine (30 mL + 70 mL + 0.1 mL). Make fresh immediately prior to use.

b. 50mM Ammonium Acetate Buffer - Use the following formula to calculate the amount of ammonium acetate needed.

mg of ammonium acetate = 50 x Liter of buffer needed x 77.08.

The ammonium acetate is dissolved in deionized water and adjusted with acetic acid to pH 4.

c. LC Mobile Phase - 70% ACN + 25% water + 5% Buffer (v/v).

Note: It is possible to shorten the retention times of the compound of interest for cleaner samples by increasing the flow rate or decreasing the % water in the mobile phase. With dirtier sample extracts, it may be necessary to change the retention time by replacing some of the water with methanol, reducing the acetonitrile level or increasing the % of water.

d. Methylene Chloride/Ethyl Acetate (3:1) - Mix one volume of ethyl acetate to three volumes of methylene chloride.

D. STANDARDS

1. Source

a. Ivermectin standard catalog no. L-640,471-076P004
Merck, Sharpe and Dhome
Rahway, NJ 07065

b. Abamectin standard catalog no. L-676-863-038A003
Merck Sharpe and Dhome
Rahway, NJ 07065

c. Doramectin standard
Pfizer
Lee-Summit, MO 64081-2998

d. Moxidectin standard-catalog no. 301423
Fort Dodge Animal Health
Division of Wyeth
P.O. Box 5366
Princeton, NJ 08543-5366

2. Preparation of Standard Solutions

a. Stock standard solution (125 μg/mL): Dissolve an appropriate amount of moxidectin, doramectin, and ivermectin separately in acetonitrile to make a concentration of 125 μg/mL.

b. Working standard solution (0.5 μg/mL): Dilute 1 mL of each stock solution (a) to 250 mL with acetonitrile in a 250-mL volumetric flask. Use this working solution as the fortification solution for recovery and standard.

3. Storage conditions:
   a. Store stock solution in freezer.
   b. Working standard solutions may be stored at room temperature.
4. Shelf Life Stability
   a. Stock: 1 year
   b. Working solution: 90 days

E. SAMPLE PREPARATION

a. Weigh, extract, and clean up the sample as described in Determinative Method (CLG-AVR), section E.a through E.f.

   Note: Fortify the recovery with the Avermectin(s) of interest or the multi-avermectin working standard at a concentration equivalent to that of the compound of interest.

b. Immediately following step E.f of CLG-AVR add 5 mL of hexane and vortex for 30 seconds.

c. Centrifuge for 3 minutes at 2500 rpm or allow to stand until layers separate (approximately 5 minutes). Remove and discard hexane top layer.

d. Evaporate acetonitrile under a gentle stream of dry nitrogen or dry air at approximately 65° ± 5° C.

e. Reconstitute the dried sample using 5.0 mL ± 50 µL acetonitrile. Vortex to mix.

f. Add 10 mL HPLC grade water and 50 µL triethylamine, and vortex for 1 minute or shake vigorously for 5 min.

g. Precondition a C8 Bond-Elut cartridge by passing 4 mL ACN followed by 3 mL SPE wash solution

h. Pass extract in aliquots through the preconditioned C8 Bond Elut SPE cartridge with 10 mL reservoir until all extracts have been added at a flow rate of one drop per second. Use a vacuum manifold to control the flow rate.

i. Rinse empty sample tube with 3 mL SPE wash and add onto the SPE cartridge.

j. Pass 8 mL of hexane through the cartridge; discard eluates. Dry SPE cartridge using a vacuum manifold for 5 minutes under vacuum (< 5 in. Hg).

k. Pre-wash the Alumina B cartridge with 4 mL methylene chloride and dry it for

l. 1 min. under vacuum (< 5 in. Hg).

m. Attach the C8 SPE cartridge above the pre-washed Alumina B SPE cartridge.

n. Elute cartridges in series with 6 mL of 3:1 methylene chloride/ ethyl acetate solution.

o. Discard eluates and C8 cartridge.

p. Draw air through Alumina-B cartridge for 30 sec.

q. Use a clean 15 mL centrifuge tube to collect eluates.

r. Attach a 10 mL Luer tip syringe to the cartridge. Pass 1.0 mL acetone through Alumina B cartridge using gravity flow collecting eluate.

s. Allow cartridge to drain and continue elution with 4 mL of methanol using gravity flow.

t. Evaporate eluate to dryness on nitrogen evaporator at 60 ± 5°C and reconstitute with 200 μL of methanol.

u. Confirm residues by injecting into a LC/APCI mass spectrometer.

F. ANALYTICAL PROCEDURE

Note: It may be necessary to optimize the parameters when different mass spectrometers are used. The key parameters include the vaporizer and capillary temperature, the voltage of the capillary and the tube lens, the resolution of the mass spectrometer, the way of tuning, and the flow rate and composition of the LC mobile phase.

1. LC/APCI Mass Spectrometer operating conditions:

a. Flow rate - 0.5 mL/min.

b. APCI probe temperature - 500°C

c. Source temperature - 140ºC

d. Ionization mode - Positive ion APCI

e. Corona discharge current - 5 μA

f. Desolvation gas flow rate - 450L/hr.

g. Cone gas flow rate - 50L/hr.

h. Tune and calibration compounds - Avermectin of interest or compounds with known spectra to cover the mass range of the ions monitored.

i. Electron multiplier voltage - 800 volts

j. Peak width at 50% peak height - 0.5μ or larger.

k. Window width of each ion - 0.3 to 0.6 amu.

l. Dwell time of each ion - 250 ms.

m. Scanning mode - Selected ion monitoring.

2. LC/MS confirmation criteria:

a. At least three characteristic ions including the molecular weight ion should be present.

b. Two or more relative abundances are ± 20% of the fortified sample (or the standard) run on the same day under the same conditions.

c. The retention time is ± 5% of the average of the standard or fortified sample.

d. The tissue blank has no confirmable target compound.

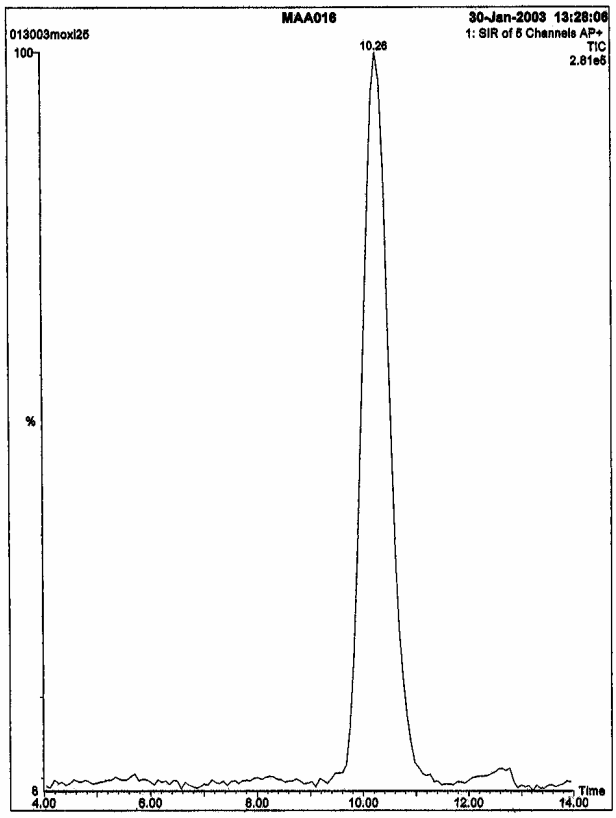
e. The signal to noise (S/N) ratio for the ions ratioed is > 3.

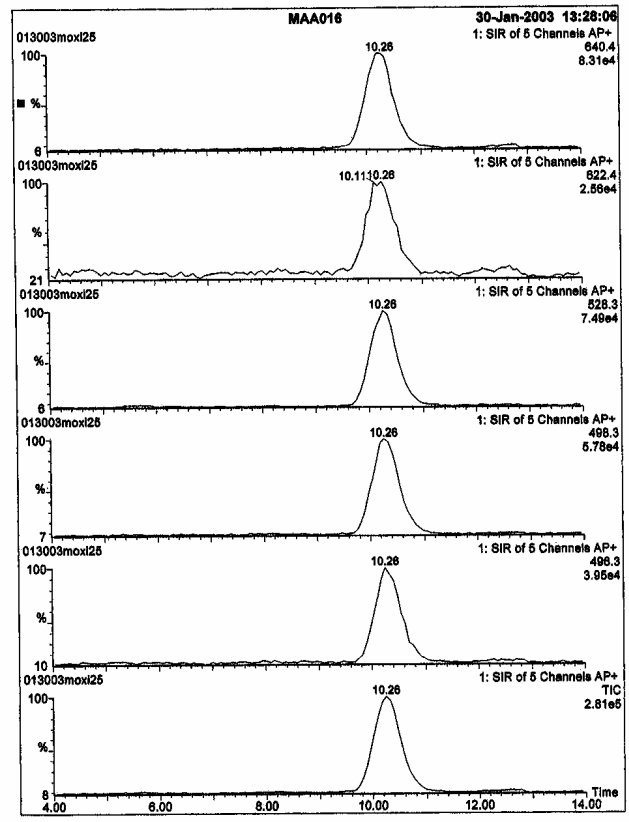
Table 1 shows the summary of fragment ions useful for confirmation of ivermectin, doramectin, and moxidectin.

Compound	**Mode**	**Ion 1 m/z**	**Ion 2 m/z**	**Ion 3 m/z**	**Ion 4 m/z**	**Ion 5 m/z**
Ivermectin	+ APCI	892.5	567.3	551.3	307.2	
	- APCI	873.5	855.5	567.3	837.5	
Doramectin	+ APCI	916.5	899.5	593.3	575.3	331.2
Moxidectin	+ APCI	640.4	622.4	498.3	496.3	528.3
Other ions may include	+ APCI	510.3	604.4	590.4		

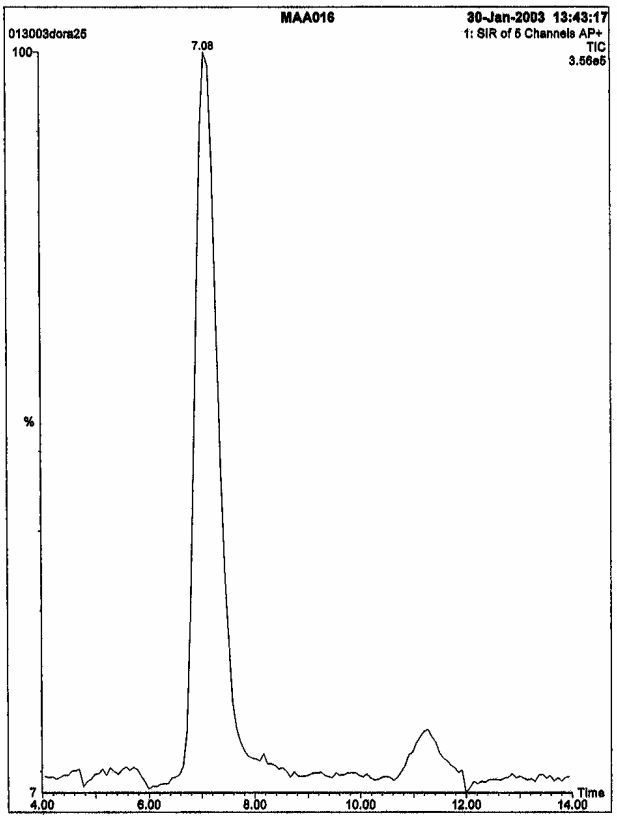
3. Sample Chromatograms

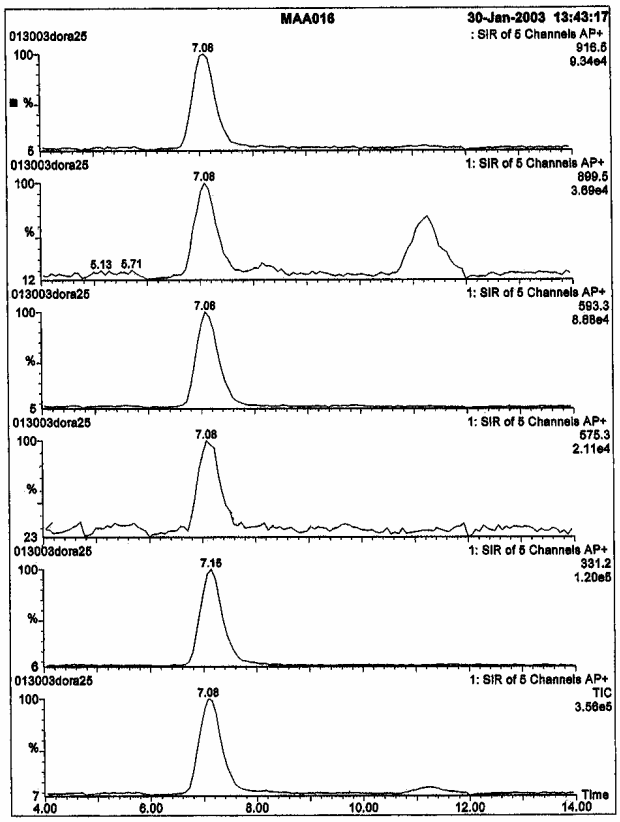
a. APCI Mass Spectra of Moxidectin standard at 25 ppb.



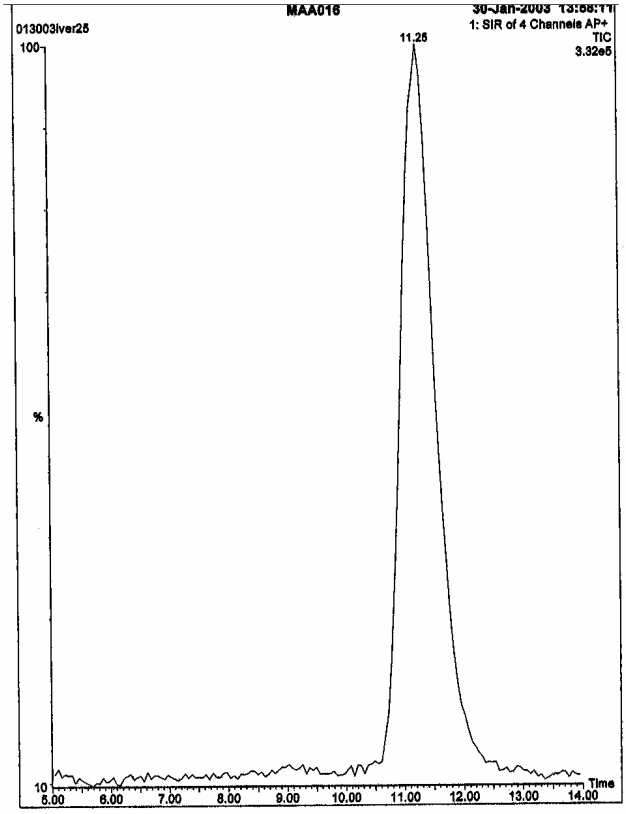


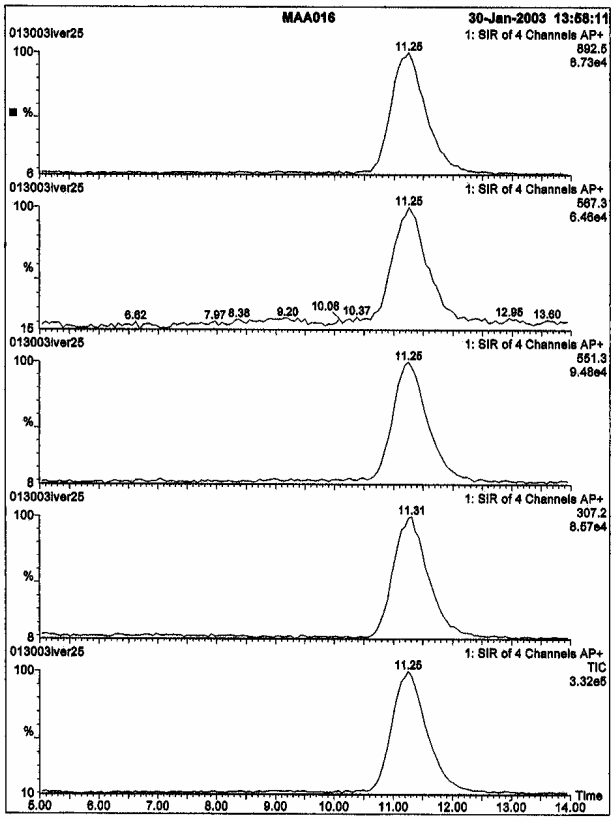
b. APCI Mass Spectra of Doramectin standard at 25 ppb.



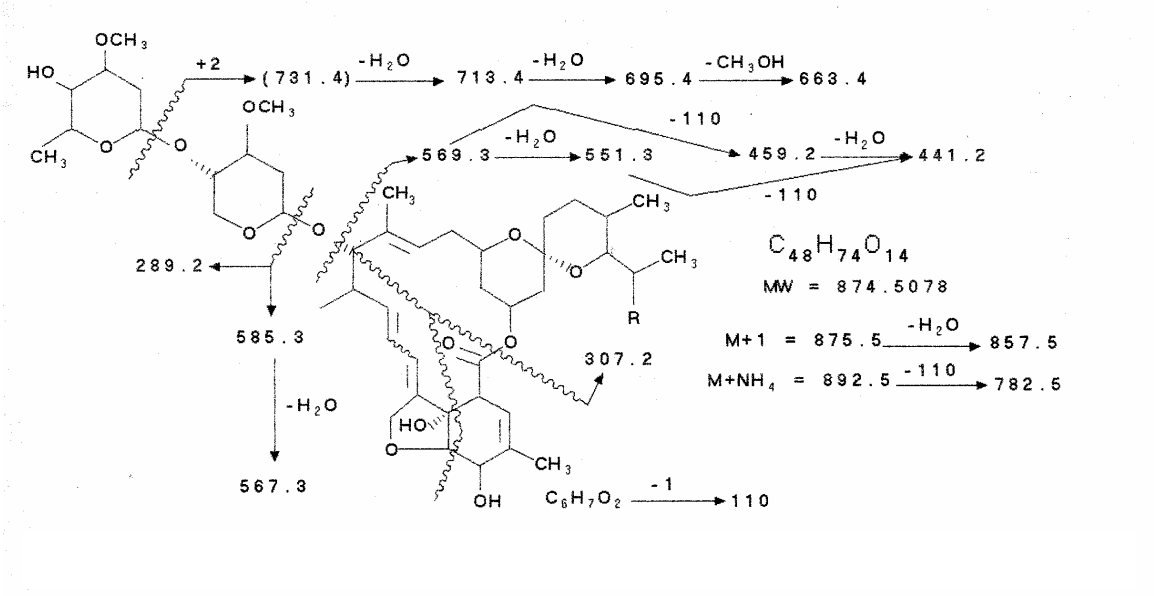


c. APCI Mass Spectra of Ivermectin standard at 25 ppb.





d. Tentative fragmentation pattern of Ivermectin.



G. CALCULATIONS (Not applicable)

H. HAZARD ANALYSIS

1. Method Title — Liquid Chromatography/Atmospheric Pressure Chemical Ionization Mass Spectrometric (LC/APCI/MS) Confirmation of Ivermectin, Doramectin and Moxidectin.

2. Required Protective Equipment — Safety glasses, appropriate gloves, lab coat.

3. Hazards

Reagents	*Hazard*	*Recommended Safe Procedures*
Acetonitrile, Methanol, Acetone, Ethyl acetate, Triethylamine, Hexane, Methylene Chloride	Flammable and corrosive, may cause skin or respiratory irritation.	Avoid contact or prolonged exposure to vapors. Work in a fume hood. Keep away from flame or heat.
Ivermectin Abamectin	Weak teratogen and possible mutagen	Handle with extreme caution.
Doramectin	Severe explosion hazard	Handle with extreme caution.
Moxidectin	May cause skin or respiratory irritation. The toxic effects of this material have not been fully evaluated.	Work in a well-ventilated area. Store material in a secure, dry, cool well ventilated room.

4. Disposal Procedures

Reagents	*Hazard*	*Recommended Safe Procedures*
Organic solvents	See above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

   a. No false positive at 0 ppb for all compounds.

   b. No false negatives at 25 ppb for all compounds

2. Critical Control Points and Specifications

Record	***Acceptable Control***
Sample weight	2.5 g. $\pm$ 0.2 g
Final dilution volume	200 μL

3. Readiness To Perform (FSIS Training Plan)

   a. Familiarization

      i. Phase I: Standards - Inject standards at the limit of confirmation and determine the ratios of the ions of interest for each component peak of compounds to be studied. Determine instrument sensitivity for each compound of interest.

      ii. Phase II: Fortified samples - For each species and tissue to be monitored, clean up and inject:

         a) A blank tissue extract

         b) Recoveries of all compounds of interest at the tolerance level or at the lowest confirmable level.

      NOTE: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

a) Analyze a total of 9 unknowns in the tissues and species to be monitored. Unknowns should be spiked as follows:

Tissue	Avermectin Spiked
Beef Liver	moxidectin, doramectin, and ivermectin
Beef Muscle	moxidectin, doramectin, and ivermectin
Horse Liver	moxidectin
Swine Liver	doramectin, and ivermectin
Swine Muscle	moxidectin
Sheep Liver	ivermectin
Sheep Muscle	doramectin, and ivermectin
Goat Liver	moxidectin
Any tissue	None detected

b) Report data to Quality Assurance Manager (QAM).

c) Letter from the QAM is required to begin analysis of official samples

b. Acceptability criteria.

Analyst must demonstrate the ability to meet confirmation criteria in section F.2 with no false positives.

4. Intralaboratory Check Samples

a. Frequency: 1 per week or as samples are analyzed.

b. Records of results are to be maintained by the analyst and reviewed by the supervisor and QAM.

c. Acceptability criteria.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

   a. Matrix: Liver and muscle

   b. Sample size: 16 oz. Minimum

6. Sample storage:

   i. Time: 6 months.

   ii. Condition: Frozen

7. Sample Set

   a. Each set should include:

      i. Tissue blank

      ii. Recovery fortified at level appropriate for the sample being confirmed.

      iii. Samples

      iv. Standard

8. Sensitivity

   a. Lowest reliable confirmation (LRC): Validated to 25 ppb.

   b. Minimum proficiency level (MPL): 25 ppb

J. WORKSHEET

An example of a worksheet, on the following page, can be removed from this book for photocopying.

AVERMECTIN CONFIRMATION

Analyst
Analyst Code
Date Started
Date Completed
Analyte of Interest
Injection Volume
Blank Tissue ILN

Critical Control Points

Sample Weight 2.5 +/- 0.2g
Final Dilution Volume 200 uL

LC Parameters

Flow Rate 0.5 mL/min
Mobile Phase 5% Buffer, 25% H2O, 70% ACN
LC Column Type Nova-Pak C-18, 60A, 4um, 2X150mm
LC Column Temp 20 C

MS Parameters

Ionization Type APCI
APCI Probe Temp 500 C
Source Temp 140 C

Std & Recovery	Ret Time	Weight	Ion Abundance Ion 1	Ion 2	Ion 3	Ion 4	Ion Ratios Ion1/Ion2	Ion1/Ion3	Ion2/Ion3

Sample	Ret Time	Weight	Ion Abundance Ion 1	Ion 2	Ion 3	Ion 4	Ion Ratios Ion1/Ion2	Ion1/Ion3	Ion2/Ion3	Std Ret Time Correlation	Rec. Ret Time Correlation	Confirmed?

Range for Standard

Ion	-20%	+20%
Ion1/Ion2		
Ion1/Ion3		
Ion2/Ion3		

Range for Recovery

Ion	-20%	+20%
Ion1/Ion2		
Ion1/Ion3		
Ion2/Ion3		

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